Development of Fluorescence Fingerprint (FF) Monitoring System for Microbial Content of Meat

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Abstract

Microbial load on beef is an important indicator that reflects the spoilage of beef. However, for industrial practice, the microbial load monitoring is conducted by aerobic plate count method whose result can be obtained after 48 hours of incubation. Thus, the conventional method is laborious, requires chemical materials and time-consuming.

Fluorescence spectroscopy has high sensitivity and selectivity to detect the trace of fluorophores components in the food matrix. The fluorescence fingerprint (FF) is a set of fluorescence spectra acquired at consecutive excitation wavelengths. FF has been used as a non-destructive technique for both qualitative and quantitative measurement. FF data are a three-dimensional data that are typically so complicated that direct interpretation is hard to understand. In this study, an analysis tool that used to interpret FF spectroscopy data is Partial Least Squares Regression (PLSR). PLSR has become a vital tool for the development of regressions between multivariate spectroscopy data and quality parameters in foods since PLSR can provide simple and robust calibrations which are applicable for future predictions.

In this study, we proposed a new method for microbial load analysis on the beef surface by the use of FF technology. In total, three different approaches were used in this study. Firstly, the microbial load was predicted from the emitted fluorophores of bacterial cells in buffer solution. Secondly, the microbial load was predicted based on metabolites secretions of bacteria. Thirdly, this FF technology was implemented by using fiber optics for the direct prediction of microbial

load on the beef surface. The main chapters of the thesis are written based on these approaches. Microbial load of *E.coli* at different dilution ranges, after a serial dilution, could be estimated by FF with $R^2 = 0.88$. The root mean square error of cross validation (RMSECV) of prediction showed that the method has a good accuracy with a low error (0.78 log CFU/cm^2). The observation of PLS regression coefficients showed that the prediction is based on intrinsic fluorescence of tryptophan that is emitted from the bacterial cell body. In addition, the prediction from the bacterial cell of the beef which was diluted with buffer solution also showed a good accuracy ($R^2 = 0.97$, RMSECV = 0.7 log CFU/cm^2). However, The Variable Importance on Projection (VIP) showed that fluorophores of metabolites trace (flavins and porhyrins) more important for the prediction than tryptophan.

In the second part of this study, we propose a novel indirect method for prediction of microbial load based on the metabolism or fluorophores trace on the meat surface. This study was conducted to elaborate an intrinsic fluorophores change on the meat surfaces.

Swab-dilute-filter (SDF) method is developed for the extraction of metabolites trace on meat surface. The surface of the meat was wiped with a sterile swab. After swabbing, the cotton swab was shaken in PBS to suspend components from beef surfaces. Some part those solutions were used for obtaining microbial load data. The rest part of solution is filtered using 0.45 µm and 0.22 µm in order to separate bacterial cells and beef residues, respectively. The filtrates that contain metabolite trace were used for FF measurements. FF as explanation variables

were obtained by fluorescence spectrophotometer F7000 (Hitachi-High-Technologies, Japan). The PLSR model was developed to estimate an aerobic plate count (APC) from FF.

The PLSR to predict APC on the surface of meat from FF had an R^2 and RMSECV of 0.88 and 0.5 log CFU/cm^2, respectively. Validation of PLSR model showed that the model was robust with RMSEP 0.52 CFU/cm^2. Microbial load prediction of beef and pork had a different VIP scores. NAD(P)H, tryptophan, and flavins are fluorophores that have high VIP sores on pork samples. On the other hand, flavins and porhyrins have high VIP score on beef samples. However, on prediction model for both pork and beef, all of the intrinsic fluorophores showed a high VIP score, which means the bacterial growth is highly related to fluorophores metabolism.

This result showed that FF had an ability to explain the microbial growth with the change of metabolites. In the other words, the result showed the microbial growth was not only directly related to the multiplication of bacterial cells but also nutrient consumption and metabolites excretions.

The third part of the research shows an appropriate Aerobic Plate Count (APC) prediction was achieved by FF coupled with fiber optics for beef samples.

FF data was obtained by fluorescence spectrophotometer F7000 (Hitachi-High-Technologies, Japan) coupled with fiber optics. The system made the measurement human contactless and suitable for industry.

PLS regression validation model for the system showed high prediction with R^2val and RMSEP of 0.813 and 0.881 log CFU/cm^2, respectively. High VIP scores are found in several wavelength regions related to four kinds of intrinsic fluorophores (Tryptophan, NAD(P)H, Porphyrins, and Flavin).

The FF method can be expected to improve monitoring technology of microbial load which can achieve rapid, nondestructive, and continuous measurement.

Contents

Figures

Tables

Chapter 1. General Introduction

1.1 Introduction

Meat and meat products have been an important part of human diet for a long time. The fresh carcass after the slaughtering can be considered as sterile, however, in the next step of post-slaughtering, contamination of meat with microorganisms can occur as the result of exposure of the animal carcass to the environment during handling, processing, or distributing (Gill, 1998). Microbial pathogens in food are found as the main cause of human illness (Hoffmann et al., 2012; Rantsiou et al., 2011; Stewart, 1987). Meat products have various amino acids and high moisture content that allowed the growth of many poisoning microorganisms (Lawrie and Ledward, 2006).

Aerobic plate count (APC) method remains the most applied and common standard by most meat producer or meat products industries. Laboratories using APC, ISO:4833:2003 as the routine determination of total viable count of food monitoring. In brief, the APC procedure can be explained as follows: sample preparation; prepare appropriate dilutions, incubating dilutions on plates at 35 ^{o}C for 48 hours, and manually counting the number of the colony. This test is simple, but can be laborious and requires chemical materials. Furthermore, this test requires almost two days to get the result. Thus, it cannot provide a fast solution for progressive food chain distribution that reaches a whole world in short time.

Spectroscopy, a rapid and non-destructive analytical method, is a good technique to solve this problem. Among several choices of spectroscopy technique, fluorescence spectroscopy gives a chance to describe related chemical transformation on the food system. Several functionally important fluorescent substances that exist intrinsically in food systems are proteins, vitamins, secondary metabolites, pigments, toxins, and flavoring compounds. This technique is also well known for its excellent sensitivity, selectivity, and rapidity.

A number of efforts to use fluorescence spectroscopy to determine meat quality had been reported. The studies cover prediction of beef toughness, texture and tenderness (Allais et al., 2004; Egelandsdal et al., 2002; Swatland and Findlay, 1997), turkey meat paleness (Swatland and Findlay, 1997), rancidity (Wold et al., 2002), and lipid oxidation (Gatellier et al., 2007; Veberg et al., 2006). Though only a few studies have reported, fluorescence spectroscopy related to microorganisms on food also had been explored, for example alteration of raw-milk cheese by *Pseudomonas spp.* (Leriche et al., 2004), microbial spoilage (Aït-Kaddour et al., 2011), and lactic acid bacteria from sausage (Ammor et al., 2004).

However, from the studies mentioned above, two main factors on microbial growth on meat were not elaborated: the intrinsic fluorophores from bacteria body itself and the transformation of metabolites during the growth. In addition, many application studies on fluorescence spectroscopy mentioned above conducted only by single excitation and emission wavelengths

which lead to loss of information from fluorescence spectroscopy to determine the quality of food systems. Therefore, it would be essential to use different excitation wavelengths and emission wavelengths simultaneously.

Fluorescence Fingerprint (FF) is one of the recent technology of fluorescence spectroscopy. FF is possible to record 3 dimensional fluorescence excitation-emission matrix for each sample. The matrix is unique for different chemical matrix, similar to fingerprint (Kokawa et al., 2012).

1.2 Objective

Based on these backgrounds, the goals of the studies were set as follows:

- To develop an estimation method of bacteria cell population by fluorescence fingerprint (FF) measurement
- To investigate the transformation of metabolites on meat surface caused by bacterial metabolism.
- To develop the microbial load prediction method based on bacterial metabolism on meat using FF
- To develop rapid, non-destructive, and contactless microbial load monitoring system by using FF coupled with fiber optics.

1.3 Spectroscopy and non-destructive analysis

Spectroscopy is an analytical technique with the use of light spectra data collections. This

analytical technique is popular because of its ability to perform rapid, less laborious, environmentally friendly, and non-destructive analysis (Scotter, 1997).

Ultraviolet-Visible (UV-VIS) reflectance, Near-infrared reflectance (NIR), Mid-infrared, and fluorescence spectroscopy are famous spectroscopy that many studies have been conducted in recent years (Cen and He, 2007; Sadecka and Tothova, 2007).

In principal, all spectroscopy has same steps (excitation, sorting, and detection). However, light wavelength, light source, and type of detection are different. As the example, UV-VIS techniques use the wavelength ranges from 200-700 nm, while near infrared spectroscopy (NIRS) using the wavelength range 700 – 2400 nm and the area from 2500-5000 nm is often referred to as the mid-infrared area and the area above 5000 nm is called as the far-infrared range (Belton, 1997; Scotter, 1997). Table 1-1 shows spectroscopy techniques with the principles employed and type of data. There are three mechanisms of the light energy after reaching the sample: light energy is absorbed by the sample, transmitted through the sample, or reflected from the sample surface. The detection step in spectroscopy is the collection of the desired data from the sample. In UV-VIS, near infrared and mid-infrared the detection is sorted by the reflection of the sample surface. In fluorescence measurements, the illumination and the reflection are optimized to specific wavelength characteristics to account for fluorophores in the sample.

1.4 FF spectroscopy

A major part of the work done during this research has been related to fluorescence fingerprint spectroscopy. This chapter will briefly go through some of the basic principles in FF spectroscopy. The description of fluorescence spectroscopy and FF will be based on Christensen et al., 2006; Fujita et al., 2010; Lacowicz, 2006; and Shibata et al., 2011.

Fluorescence is the emission of light as a result of processes that occurs in molecules. Some molecules, especially polyheterocycles can be excited by absorption light to a higher energy stated or called excited state. A molecule with high energy state could not sustain for long, the energy will decrease resulting release amount of light energy, and this process is fluorescing.

A fluorophore is a molecule that is capable to fluoresce. At ground state the fluorophores have relatively low energy and stable configuration, they are not fluorescing. When light from external sources is radiated to a molecule, the molecule could absorb the energy. If the energy is sufficient the molecule goes to higher energy state, this process is called excitation. Emission is a process when the fluorophore will rearrange from excited state back to the ground state level energy and the excess energy release as the emitted light.

The general principles of fluorescence steps can be illustrated by a Jablonski diagram, as shown in Figure 1-1.

Excited singlet state followed by a vibrational relaxation or internal conversion, where the molecule undergoes a transition from an upper electronically excited state to a lower one. Finally, the emission occurs, typically 10^{-8} seconds after the excitation, when the electron returns to its more stable ground state, emitting light at a wavelength according to the difference in energy between the two electronic states. The fact that fluorescence is characterized by two wavelength parameters (excitation and emission), fluorescence spectroscopy significantly has improved specificity, compared to spectroscopic techniques based only on absorption (Karoui and Blecker, 2010).

According to the Jablonski diagram Figure 1-1, the energy of the emission is lower than that of excitation, meaning that the fluorescence emission occurs at a longer wavelength than the absorption (excitation). The difference between the excitation and emission wavelength is known as Stoke's shift (Valeur and Brochon, 2001). The Stokes shift, which, in contrast to absorption spectroscopy, allows for emission of photons to be detected against a low background, combined with efficient detectors in the visual range makes fluorescence spectroscopy a very sensitive analytical method with possibilities to measure down to parts per billion levels (Christensen et al., 2006; Lacowicz, 2006).

Foods contain fluorescent compounds such as aromatic amino acids, vitamins and cofactors, nucleic acids, porphyrins, flavonoids, coumarins, alkaloids, and mycotoxins and aflatoxins

(Wehry, 1986). Excitation and emission peak of food relevant single fluorophores can be found at fluorescence library. Table 1-2 shows some fluorophore from web-base library at www.models.kvl.dk .

Normally, only emission or excitation spectra (i.e. one excitation or emission wavelength) are recorded when investigating the fluorescence of a sample (Fig. 1-2). The wavelength of the emission peak is used for qualitative analysis (determination of the constituents which compose the sample) and the height of the peak for quantitative analysis (determination of the amount of the constituent). Instead of one emission spectrum from one excitation wavelength, multiple emission spectra can be acquired by irradiating the sample with a set of consecutive excitation wavelengths.

The use of only a set of excitation and emission wavelengths could limit the ability of fluorescence spectroscopy to determine the quality of food systems (Karoui and Blecker, 2010). In order to completely determine several compounds on food, the use of different excitation wavelengths is necessary.

FF is the excitation and emission properties that presented as fluorescence landscape as shown in figure 1-3. It is acquired by measuring the fluorescence intensity of a sample at consecutive excitation and emission wavelengths (Karoui and Blecker, 2010).

Unlike the conventional fluorescence spectrum which focuses mainly on the spectrum peak,

the FF makes use of the whole pattern, taking in consideration the fluorescence intensity of wavelengths other than the peak point. This means that FF can discriminate between samples which have a similar dominating component but have differences in minor components, for example, fruits of the same variety cultivated in a different region.

The pattern of the FF diagram, like a fingerprint, shows the fluorescence characteristics of each constituent in a sample, thus making the use of FF suitable for identifying certain constituents and detecting slight differences between samples (Tsuta et al., 2007).

The FF spectra of beef are depicted in Figure 1-3. On this figure X axis is emission wavelength while Y axis is excitation wavelengths. The peak of spectra with intensity 6000 arbitrary units (a.u) is described on the yellow region. Two diagonal line on figure 1-3 could be considered as Rayleigh scatter. Rayleigh scatter refers to the scattering of light by particles and molecules smaller than the wavelength of the light. Rayleigh is so-called elastic scatter, meaning that no energy loss is involved, so the wavelength of the scattered light is the same as that of the incident light. Due to the construction of grating monochromators used for excitation light in most spectrofluorometers, some light at the twice longer emission wavelength than the chosen excitation wavelength will also pass through to the sample. For this reason, an extra band of Rayleigh scatter, so-called second-order and third-order Rayleigh, will typically appear in fluorescence measurement at the twice longer emission wavelengths than the given excitation

wavelength.

On this work, FF spectroscopy is coupled with fiber optics in order to match the necessity for monitoring at the online condition. Fiber optics offer advantages such as simple preparation procedure, relatively fast response, wide response range, reasonable selectivity and high sensitivity. Optical fiber based techniques which can be used for a variety of different sensor purposes, providing a foundation for an effective measurement technology, which can complete with conventional methods, usually in niche areas. Therein lies the recipe for the success of optical fiber sensors — in tackling difficult measurement situations where conventional sensors are not well suited to use in a particular environment. The resulting sensors have a series of characteristics that are familiar: they are compact and lightweight — in general, minimally invasive — and fiber sensors offer the prospect that they can be multiplexed effectively on a single fiber network (Grattan and Sun, 2000).

1.5 Partial Least Squares Regression

Data from spectrophotometric instruments are typically so complicated that direct interpretation is hard to understand. Hugh three-dimensional data, overlap peaks, shifted peaks are example problems to mention as the complexity of spectroscopy data looks like. That is why chemometric methods need to be applied for the data to be analyzed effectively.

Chemometric methods are mathematical and statistical methods which decompose complex

multivariate data into simple and easy-to-interpret structures that can improve the understanding of chemical and biological information. Chemometrics has been defined as the "chemical discipline that uses mathematics, statistics and formal logic (a) to design or select optimal experimental procedures; (b) to provide maximum relevant chemical information by analyzing chemical data; and (c) to obtain knowledge about chemical system" (Massart et al., 1988).

In this thesis, part of chemometrics that used to analyze spectroscopy data is partial least squares regression (PLSR). Explanation of theoretic PLSR in this thesis are based on textbooks from Beebe et al., 1998; Massart et al., 1988; Næs et al., 2002; Sharaf et al., 1986.

PLSR has become a vital tool for the development of regression models between multivariate spectroscopy data and quality parameters in foods, since PLSR can provide simple and robust calibrations which are applicable for future predictions.

The strategy of PLSR is to reduce the dimension of the spectroscopy data (X) and y space by creating linear combinations of the original variables. These new (latent) variables or components are statistically independent and ideally carry all relevant information. The reference variable, e.g. bacterial content, to be predicted is used actively in determining these components, and a linear regression model is defined as

$$\boldsymbol{y = Xb + E} \qquad (1)$$

where *b* is the corresponding vector of regression coefficients, and *E* their residuals (model errors, noise and so on). In order to obtain a good estimation of b, the PLSR model needs to be calibrated on samples which span the variation in *y* well and in general are representative of the future samples.

Validation of the chemometric models is very important in order to determine the correct number of latent variables so that noise is not included in the model, to detect outlier and to obtain reliable estimates of prediction error. In this thesis cross-validation and a validation with an external data set were applied.

Cross-validation is used when the number of samples in a dataset is limited and all samples therefore are needed for both calibration and validation. Samples data is divided into some segments. One by one each segment is left out and the model is calibrated on the remaining samples and used to predict the samples in the left out segment. There are several different cross-validation methods, in this research Venetian Blinds cross-validation is used. In Venetian Blinds cross-validation test set is determined by selecting every s^{th} object in the data set starting at objects numbered 1 through *s*. The estimation of error is expressed as root mean square error of cross-validation (RMSECV).

Cross-validation allows an estimation of the performance of a model when it is applied to

unknown data. One of the data sets is used for calibration, while the other is used for validation. Since different samples are used for calibration and validation, the result is a stronger test of a model and estimation of error (Root Mean Square Error of Prediction-RMSEP) will usually be closer to the true value than RMSECV.

The measure of model performance is usually given by the correlation coefficient (r), which is the correlation between the measured reference (y) and the predicted reference ($\hat{y}$), and by the prediction error RMSECV (root mean square error of cross-validation) or RMSEP (root mean square error of prediction):

$$RMSECV \text{ or } RMSEP = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(y_i - \hat{y}_i)^2} \qquad (2)$$

where $\hat{y}_i$ is the predicted value for sample i, y_i is the corresponding reference value, and N is the total number of samples.

Table 1-1. Type of spectroscopy and their principles (Scotter, 1997)

Spectroscopy technique	**Wavelength range**	**Information obtained**
UV-VIS	UV and visible	Colour/fluorescence
Photoacoustic	Near-infrared and mid infrared	Protein, fat, water content
Optothermal	Near-infrared and mid infrared	Protein, fat, water content
Near-infrared	Organic bond types and physical structure	Bond vibrations
Raman	Near-infrared and mid infrared	Bond vibrations
Mid-infrared	Mid-infrared	Bond vibrations
Nuclear magnetic resonance	Radiowaves	Change of nuclear spin

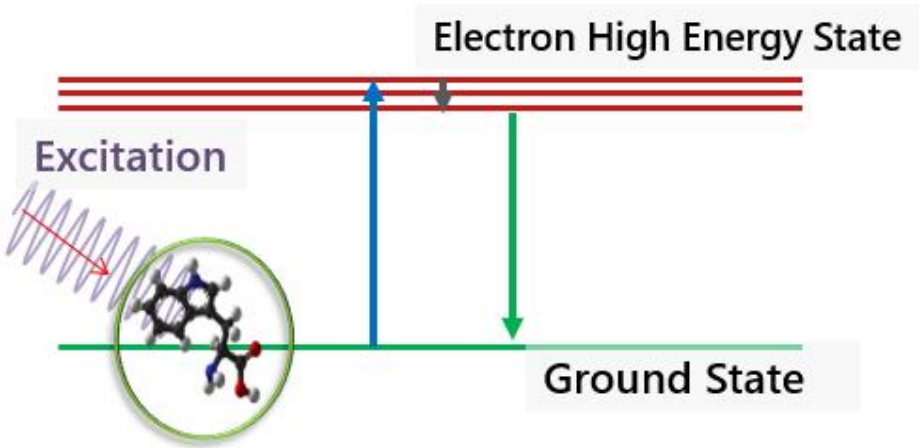

Figure 1-1. Jablonski diagram showing the basic principles in fluorescence

Table 1-2. List of 11 Food-Relevant fluorophores and their fluorescent properties (Christensen et al., 2006).

Fluorophore	Excitation λ_{max} (nm)	Emission λ_{max} (nm)
phenylalanine	258	284
tyrosine	276	302
tryptophan	280	357
Vitamin A (retinol)	346	480
Vitamin B_2 (riboflavin)	270(382,448)	518
Vitamin B_6 (pyridoxin)	328	393
Vitamin E (α-tocopherol)	298	326
NADH	344	465
ATP	292	388
Chlorophyll *a*	428	663
hematoporphyrin	396	614

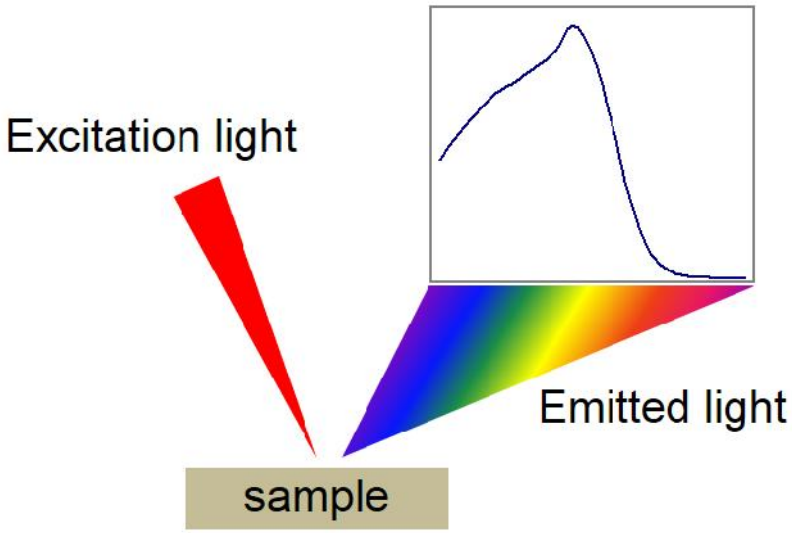

Figure 1-2. Conventional fluorescence spectroscopy

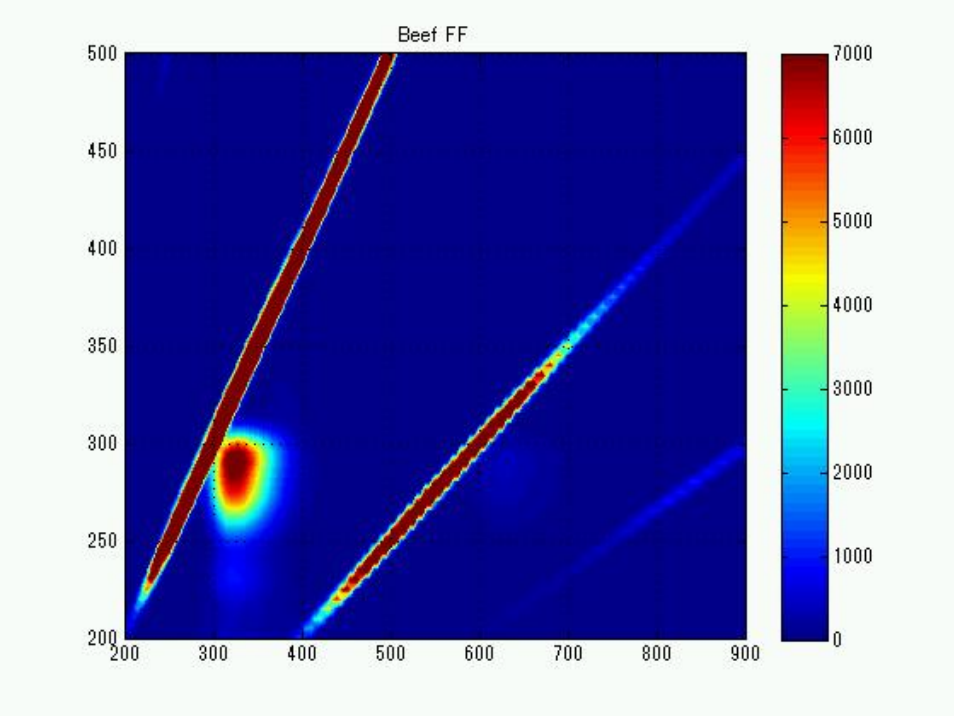

Figure 1-3. Fluorescence Fingerprint (FF)

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Chapter 4. Fiber Optics Fluorescence Fingerprint Measurement for Aerobic Plate Count Prediction on Sliced Beef Surface

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4.1 Introduction

Modern food chain distribution requires less time to deliver fresh foods to consumers. Nevertheless, care should be taken to avoid contamination with microorganisms which could result in severe food-borne outbreaks (Sodha et al., 2011; Walsh, 2013). In particular, microorganisms in meat pose the most severe threat to human health. Pathogenic bacteria such as *Salmonella spp*, *Escherichia coli*, and *Campylobacter spp.* are well known microorganisms of meat that could be a source of food-borne outbreaks (Kinross et al., 2014; Koutsoumanis et al., 2006; Matulkova et al., 2013; Schneider et al., 2011). Therefore, a rapid, nondestructive, and quantitative method of microbiological analysis is essential for meat-production monitoring. However, conventional methods for monitoring microorganisms such as aerobic plate counts (APCs) by the culture method, are time-consuming and laborious.

Spectroscopy is a rapid and nondestructive technique which can be used to solve this problem. Among the several spectroscopic techniques, fluorescence spectroscopy has better sensitivity and selectivity than absorption spectroscopy (Karoui and Blecker, 2010). Fluorescence fingerprint (FF) is a set of fluorescence spectra acquired at consecutive excitation wavelengths.

FFs have been used as a nondestructive technique for both qualitative and quantitative food assessment for mycotoxins (Fujita et al., 2010), gluten, starch (Kokawa et al., 2012), and buckwheat content (Shibata et al., 2011).

Several studies on employing fluorescence spectroscopy to determine meat quality have been reported. These studies cover the prediction of beef toughness, texture, and tenderness (Allais et al., 2004; Egelandsdal et al., 2002; Swatland and Findlay, 1997), turkey meat paleness (Swatland and Findlay, 1997), rancidity (Wold et al., 2002), and lipid oxidation (Gatellier et al., 2007; Veberg et al., 2006). Among them, a few studies on fluorescence spectroscopy in relation to microorganisms in food, such as the alteration of raw-milk cheese by *Pseudomonas spp.* (Leriche et al., 2004), microbial spoilage (Aït-Kaddour et al., 2011), and lactic acid bacteria from sausages (Ammor et al., 2004), have been reported.

There are two major drawbacks to the above studies related to fluorescence spectroscopy. Firstly, in many studies only a single set of excitation and emission wavelengths was used, leading to a loss of information when using fluorescence spectroscopy to determine the quality of food systems. Therefore, it would be interesting to measure fluorescence at different excitation wavelengths and emission wavelengths to simultaneously collect information on several constituents. Secondly, many measurements were conducted inside the chamber of a fluorescence spectrophotometer, which is not suitable for at-line analysis. FF spectroscopy with fiber optics is a potential technique for overcoming these problems. FF spectroscopy coupled

with multivariate analysis appears to be applicable to the nondestructive determination of the APC on the surface of lean beef (Yoshimura et al., 2013). However, in their study, the sample holder, which was made from quartz glass, was in contact with the surface of the lean beef. Sampling that requires contact with samples is undesirable for at-line monitoring because contaminated devices could be a source of cross-contamination (Davies and Board, 1998). Thus, applications of FF which is contactless and can be measured outside the fluorescence spectrophotometer is still unreported. A breakthrough in solving this sampling problem could be achieved by coupling FF measurement with fiber optics. Fiber optics is an outgrowth of the communication industry and allows the transmission of light over long distances with high efficiency (Daneshvar et al., 1999).

Therefore, the objective of this study was to develop an estimation method for the APC on the surface of beef by FF measurement through fiber optics and multivariate analysis. Thanks to fiber optics, FF spectroscopy was successfully applied to sliced beef without any contact and was conducted outside the fluorescence spectrophotometer.

4.2 Material and methods

4.2.1 Sample beef preparation

Beef slices were purchased from a local meat retailer and transported to the laboratory of the

National Food Research Institute, National Agriculture and Food Research Organization (NARO) in Ibaraki, Japan. Beef samples were cut at the store on the starting day of storage, to a cut size of 50×50 mm per slice and a thickness of 8±1 mm. The samples were then stored in a refrigerator at 15 ℃ during experiment periods (72 h). During storage, the samples were placed in sterilized plastic Petri dishes with lids. In the beginning of the experiment, a total 35 beef slices on petri dish were prepared, the 24 beef slices will be measured and the rest are a spare. The spare samples were prepared in order to avoid rough surface of beef. Thus, an average equal distance between fiber optics and meat surface could be obtained. Fig. 4-1 shows a flowchart of the experiments. Two independent experiments were conducted with same procedure on this study.

4.2.2 FF measurements

FF measurements were performed on beef slices using a fluorescence spectrophotometer (F-7000, Hitachi High-Technology Corp., Tokyo, Japan) with fiber optics (5J0-0114-F-7000, Hitachi High-Technology Corp., Tokyo, Japan). The fluorescence spectrophotometer equipped with a 150 W Xe arc lamp as the light source and a grating monochromator as the wavelength selector. The scan speed was set to 60000 nm/min. Consequently, the measurement time is 2 minutes. As shown in Fig. 4-2, the system consisted of a spectrophotometer, fiber optics, and an XY stage controller. The excitation and emission wavelength ranges for FF measurement

were 200 – 500 nm and 200 – 900 nm, respectively, both with wavelength intervals of 5 nm. A total 2 minutes of measurement. A 500×500×500 mm box was placed outside the fluorescence spectrophotometer and used as a darkroom for the measurement. The box walls were coated with a black cloth to prevent exposure to the outside light. Excitation light was transferred through the fiber optics to the darkroom where the sample was placed. The emission signal from the sample surface was captured by the same fiber optics then sent to the detector of the fluorescence spectrophotometer. The fiber optics used for excitation were placed perpendicular to the light source, while the fiber optics used for emission were perpendicular to the detector of the fluorescence spectrophotometer. A Y-shaped joint was used to bundle both the excitation and emission fiber optics in a single casing. The random arrangement of excitation and emission fiber optics at the end of the probe was oriented toward the sample at distance of 3 – 5 mm.

A Petri dish containing the beef sample was placed on an XY stage controller inside the darkroom under the fiber-optic probe. The XY stage controller was used to move the sample to measure the FF signal from nine points on the sample surface. The movement of the XY stage controller was controlled using a program developed by system development software (Labview 8.6, National Instruments Inc., USA).

The FF measurement and microbial counts were performed after 12, 24, 36, 48, 60, and 72 h of storage. Two slices were measured for each storage time.

4.2.3 Microbiological analysis

The nine points used in the FF measurement on beef sample surfaces were wiped with a sterile swab after the measurement. An area of 1×1 cm^2 was swabbed with one cotton swab kit (GSI Creos Corp., Japan). To ensure valid sampling, the area was swabbed in a horizontal pattern and again in a vertical pattern. This allowed the entire surface to be swabbed evenly (Bautista et al., 1997; Oto et al., 2012). After swabbing, the cotton swabs were placed into its container that contained 10 ml of 0.1% peptone to be used for plate counts.

Serial decimal dilutions were prepared with Phosphate Buffer Saline (PBS), and the diluted samples were pour plated (1 ml) in duplicate on standard agar (Nissui, Japan). Samples with a high range of bacterial load were plated using an Eddy Jet Spiral Plater (IUL, Japan). The APC was determined by counting colonies after 48 h of incubation at 35 ^{o}C. A total of 216 (nine points/sample × six storage time × four samples/storage time) APC were determined in the two experiments.

4.2.4 Fluorescence fingerprint data analysis

4.2.4.1 FF data extraction

The extraction of FF data and preprocessing were performed in accordance with a previous

study (Fujita et al., 2010). The attached software FL Solutions (Hitachi High-Technology Corp., Tokyo, Japan) was used to acquire the data. The original FF data consisted 8601 recorded intensities for one-point measurement. The FF data were preprocessed and then unfolded from 3D to 2D (Guimet, 2004; Obeidat et al., 2007) with Microsoft Office Excel 2007 (Microsoft, USA) and Matlab 2007b (MathWorks, Inc., USA). The preprocessing procedure included (a) the removal of data with emission wavelengths shorter than the excitation wavelength, because fluorescence data always comes from emission wavelengths longer than the excitation wavelengths. (b) The removal of the scattered light and second, third, and fourth-order light. These were generated by light scattering from the surface of the diffraction grating, were not fluorescence. (c) The removal of data with excitation wavelengths lower than 250 nm and emission wavelengths higher than 850 nm, for which significant noise was observed (Fujita et al., 2010; Shibata et al., 2011). A total of 3991 FF intensities obtained by one-point measurement remained after the preprocessing procedure.

4.2.4.2 Partial least square regression analysis

Partial least squares regression (PLSR) was applied to develop a model for predicting the APC of the beef using FF data as explanatory variables. In total, 108 (two samples/storage time × nine points/sample × six storage times) APC and FF data values were used as the calibration set, and the same amount of data was used as test data for validation. All the data

were transformed to logarithmic values and then normalized and mean-centered. The optimization of latent variables (LVs) was carried out by venetian blind cross-validation (Davis et al., 2006). The number of LVs was chosen by searching for the local minima and the knee of the root mean square error of cross-validation (RMSECV) drop curve. The prediction accuracy was evaluated by observing the RMSECV, the root mean square error of prediction (RMSEP) and the coefficient of determination (R^2). MATLAB 2007b (MathWorks, Inc., USA) along with PLS Toolbox 6.7.1 (Eigenvector Research, Inc., USA) were used to develop the PLSR model. At the same time, the variable of importance projection (VIP) score and the coefficient of the PLSR model were calculated.

While the PLSR model used numerous FF variables for predicting the APC, the important variables for the prediction using the PLSR model could be obtained by evaluating the VIP and regression coefficient (Wold et al., 2001). The VIP score indicates the relative influence of each independent variable and is the sum of the effect of each variable's over all the model dimensions divided by the total explained variation obtained by the model (Trap et al., 2013). On the other hand, the sign of the regression coefficient indicates the direction of the correlation between the APC and the corresponding variable.

4.3 Results and discussion

4.3.1 Aerobic plate count

Figure 4-3 shows the microbial load of beef slices during storage under an aerobic condition at 15 ^{o}C. The exponential growth phase occurred between 12 and 72 hours. During this phase of growth, not only was the microbial load was increasing but (bio)chemicals on the meat surface were also altered (Madigan et al., 2010). The microbial load in the first 12 hours of the first experiment was an average of 4.8 log CFU/cm^2. This number increased every 12 hours until spoilage of the sample, that is, intense discoloration and the presence of off-odours, was observed at 48 hours with a microbial load of approximately 8.46 log CFU/cm^2. In contrast, the microbial load in the first 12 hours of the second experiment was about half that in the first experiment. Moreover, in the second experiment, the beef sample was spoiled after 60 hours of storage with a microbial load of approximately 6.89 log CFU/cm^2. The samples in the first and second experiments had different initial loads. Prior to slaughter, the meat from a healthy animal is considered to be sterile. It is through the processes of immobilization, exsanguination, hide removal, evisceration, handling, and further processing into wholesale and retail cuts that bacteria may come in contact with beef tissue (Nollet, 2008). The difference in the initial load in the two experiments may be related to differences in these handling processes.

4.3.2 Fluorescence fingerprints

The FFs of beef slices stored for 12 hours in the first experiment are shown in Fig. 4-4. The highest fluorescence intensity was observed at an excitation wavelength of 295 nm and an emission wavelength of 335 nm. These highest intensities could be contributed by tryptophan (Leblanc and Dufour, 2002), which is an amino acid contained in meat. Although the FFs were measured using fiber optics, they showed a similar pattern to those reported in other studies (Oto et al., 2012; Yoshimura et al., 2013), where the conventional measurement of FFs was conducted inside the chamber of a fluorescence spectrophotometer.

Although the full dynamic range in the fluorescence spectrophotometer was 0 – 10000 a.u., Fig. 4-4b shows the fluorescence intensity plotted on a logarithmic scale. On this scale, the fluorescence intensity of porphyrins (excitation = 430 nm, emission = 590 and 660 nm) can be observed (Christensen et al., 2006; Ramanujam, 2000). This fluorescence peak (region B) can be attributed to either microorganism or animal cells, which excrete small amounts of porphyrins (Doss and Philipp-Dormston, 1971).

4.3.3 Partial least square regression of FF data

Table 4-1 shows the results of PLSR and the number of LVs for different preprocessing methods. Before developing the PLSR model several preprocessing techniques were applied to raw FF. Mean center and auto scale are common preprocessing methods, both transformed the data by scaling method. Normalization is a preprocessing procedure with the objective of

reducing systematic differences between observations due to, for example, variation in sample concentration or in analytical sensitivity (Bylesjö et al., 2009). Mean center scales the intensity at each wavelength to zero mean, while auto scale scales it to zero mean as well as unit variance. Venetian blind was used as cross validation method. In this method each test set is determined by selecting every stratified object in the dataset. The algorithm of local minima suggested a PLS model with 5 LVs, while the results of the find knee procedure suggested the suitability of the model with 6 LVs. The optimum prediction model and the best preprocessing method were concluded on the basis of the observation of R^2 during model development using the calibration set and R^2 during the model testing using the validation set and RMSEP. Preprocessing of the FF data by normalization followed by mean centering was found to be the best prediction model (LV=6) with a prediction error of 0.831 log CFU/cm^2. This result shows systematic differences on beef sample due to variation in beef surface and in APC method sensitivity were reduced by normalization.

The relationship between the conventional measured APC analysis and the predicted APC obtained using the FF with fiber optics are shown in Figures 4-5 and 4-6. The prediction model for the APC was constructed with six LVs, which gave the best result with the highest coefficient of determination and the lowest RMSECV (R^2 = 0.983; RMSECV = 0.835 log CFU/cm^2). For validation of the model, a new external dataset contain 108 data of FF were used by the model for APC prediction. The RMSEP obtained from the validation expresses the

average error expected in future predictions. In the validation, a strong correlation ($R^2 = 0.859$) and a small prediction error (RMSEP = 0.831 log CFU/cm^2) were obtained. The prediction accuracy of the regression model based on the FF fiber-optic dataset was thus verified.

4.3.4 VIP score and regression coefficient of the PLSR model plotted using FF contour representation

Figure 4-7 shows the VIP score and the PLSR coefficient plotted using a contour representation. A VIP score of more than 1 indicates the important regions in the model. Moreover, a higher score indicates more significant variables (Wold et al., 2001). Although VIP scores can be used to mark the important regions, they do not show whether the correlation between the corresponding variables and the APC is positive or negative. Therefore, Fig. 4-7b shows the distribution of the PLSR coefficient along with the sign of regression coefficient obtained from the FF data. The regions related to four types of fluorophores on the beef surface show high VIP scores and regression coefficients for the prediction of the microbial content.

The VIP score and regression coefficient of tryptophan fluorophores correspond to region A in Fig. 4-7. This region has a high VIP score with a negative regression coefficient. Considering these values, the FF intensities of tryptophan decreased with increasing microbial content. The decreasing is related to amino acids as the energy sources of bacteria. Almost all the bacteria comprising the meat microflora grow by catabolizing low-molecular-weight compounds of

meat such as glucose, lactic acid, amino acids, nucleotides, urea, and water-soluble proteins (Gill, 1986). It is has been established that there is an order in which these compounds are catabolized by the major organism causing meat spoilage, namely, the first main energy source is glucose, the second is lactate, and the third is amino acids (Nychas et al., 2007). The degradation of amino acids including tryptophan is known to start shortly before the onset of spoilage due to glucose exhaustion (Gill, 1986). Therefore, region A corresponding to tryptophan was considered to have a high VIP score with a negative correlation because of the degradation of amino acids including tryptophan by microorganisms on the beef surface.

A positive regression coefficient comes from the emission of porphyrins (Fig. 4-7, region B). Porphyrins are a large group of organic compounds that consist of four pyrrole rings joined by methane bridges. Porphyrins fluorophores are found in many experiments on food such as dairy products (Wold et al., 2005) and meat (Ashby et al., 2003; Veberg et al., 2006; Wakamatsu et al., 2004). The fluorescent porphyrin species investigated in previous studies on meat and meat products were protoporphyrin IX (PP) and zinc protoporphyrin (ZnPP), the fluorescence of PP and ZnPP intensities in fresh pork meat increase with storage time and temperature. (Schneider et al., 2008). The shelf-life prediction of meat and meat products under different storage conditions can also be derived from the fluorescence of phorphyrins (Durek et al., 2012). Porphyrins on a meat surface are formed by the activity of microorganism (Doss and Philipp-Dormston, 1971). In addition, the amount of porphyrins synthesized by bacteria depends on

the species and the ambient conditions (Doss and Philipp-Dormston, 1971; Harris et al., 1993). For example, during frozen storage, no porphyrins form or are decomposed because of the low rates of microbial growth and enzymatic processes (Durek et al., 2012). Thus, the similar behavior of porphyrins fluorescence spectra to that in previous studies was considered to result in the positive PLSR coefficients and the high VIP score in this study.

Region C in Fig. 4-7 correspond to excitation at about 320 nm and emission at about 440 nm. This combination could be attributable to the contribution of the NAD(P)H fluorescence peak from bacteria. Several studies have suggested that NAD(P)H fluorescence could be a fingerprint of bacteria (Leblanc and Dufour, 2002; Sahar et al., 2011; Tourkya et al., 2009). NADH fluorescence can be used to classify bacterial species (Leblanc and Dufour, 2002; Tourkya et al., 2009), and an emission band shift can be observed among some species of *Pseudomonas*, *Stenotrophomonas*, *Xanthomonas* and *Burkholderia* (Tourkya et al., 2009). The positive PLSR coefficient in the NAD(P)H region can be an indication that our FF fiber-optic system recorded the NAD(P)H of bacteria.

Region D in Fig. 4-7 (Ex/Em = 420/520) shows the contribution from phospholipid metabolism and bacteria. In a previous study, fluorescence spectra as promising tools for the measurement of lipid oxidation (Gatellier et al., 2007; Veberg et al., 2006); Veberg et al., 2006); Veberg et al., 2006)). On the other hand, some bacteria could be successfully identified fluorescence spectra with an excitation wavelength of 410 nm (Giana, Silveira Jr., Zângaro, & Pacheco,

2003). Thus, the positive PLSR coefficients in this region may be related to the growth of bacteria on the meat surface.

The growth of microorganisms on food is a complex event, in which a combination of microbial and biochemical activities may interact. It is still a major task to find a relation between the microbial composition and metabolites applicable to the evaluation and the possible prediction of microbial spoilage (Huis in't Veld, 1996). In this study, the plots of the VIP score and PLSR coefficients show important regions, some of which resemble the fluorescence profiles of intrinsic fluorophores and bacterial load, which may enable us to describe the interaction between the microorganisms and biochemical changes in meat.

4.4 Conclusions

An FF spectroscopy system with fiber optics was able to predict the APC on a beef surface with prediction error of 0.831 log CFU/cm^2. The FFs may have been emitted from intrinsic fluorophores, namely, tryptophan, NAD(P)H, porphyrins and phospholipids.

The PLSR model developed on the basis of the FFs was practical, because the calculated results reflected the sequential reactions on the beef surface. Thus, the growth of microorganisms on meat can be described not only by an increase in the number of bacteria but also by changes in the components on the surface of the meat.

The simple sample preparation and the ability to measure FFs outside the fluorescence

spectrophotometer chamber using the fiber-optic system are considered to be advantageous for monitoring the bacterial content under at-line conditions.

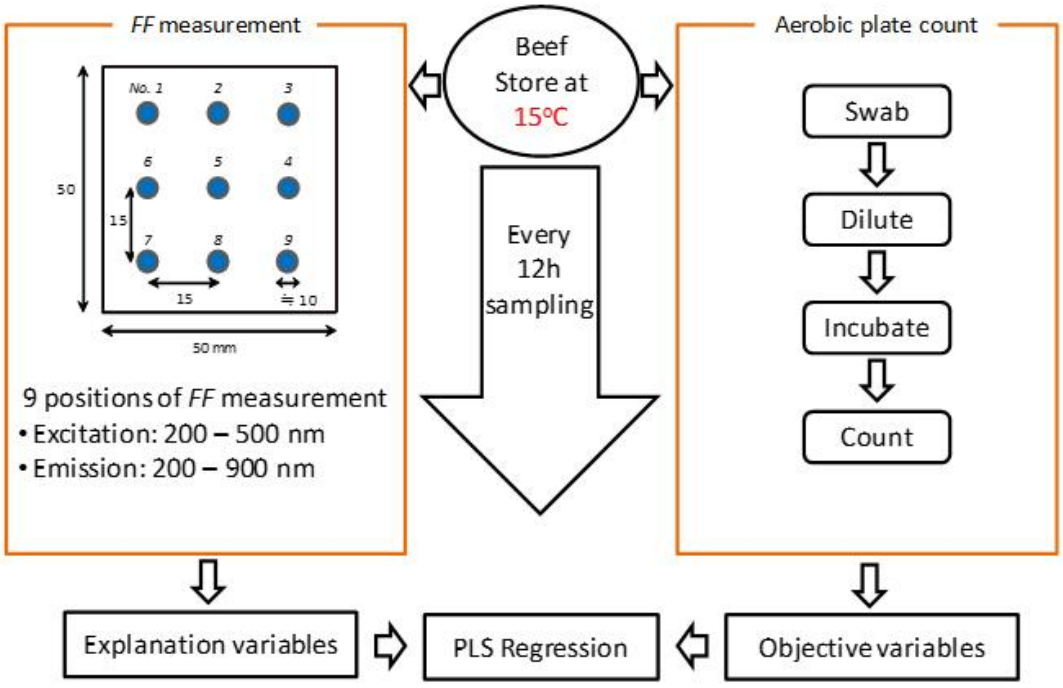

Figure 4-1. Flowchart for FF measurement through fiber optics experiment

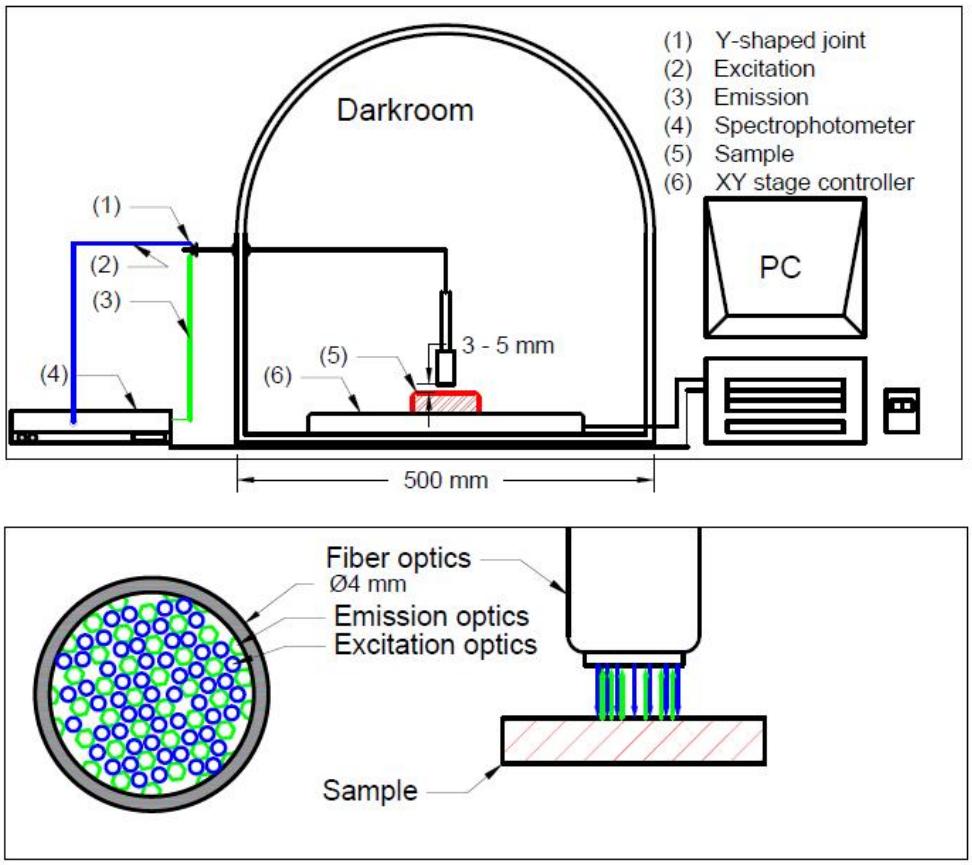

Figure 4-2. Schematic structure of FF with fiber optics system

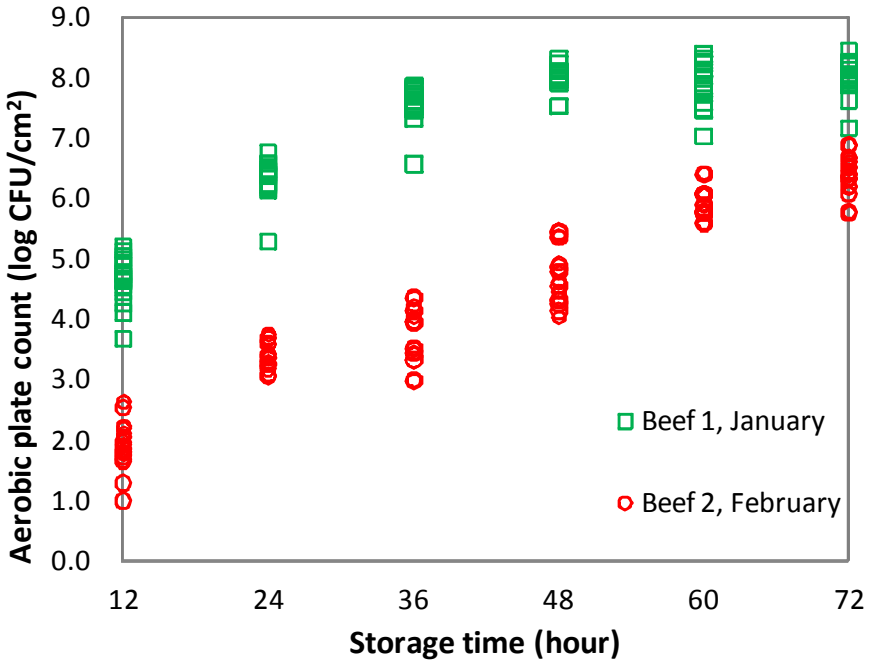

Figure 4-3. Aerobic plate count of beef slices during storage

□ Experiment 1;

o Experiment 2

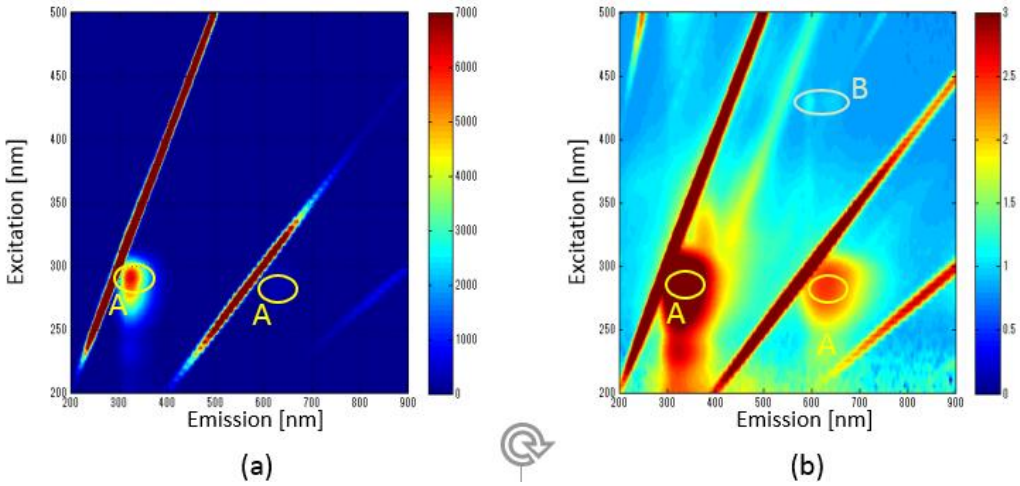

Figure 4-4. Fluorescence fingerprints (FFs) of beef (12 h) obtained using fiber optics

(a) FF of beef surface in range of normal intensities (0 – 7000 intensity (a.u.)).

(b) FF of beef surface shown with log-scale intensities.

A: regions related to tryptophan; B: region related to porphyrins.

Table 4-1. Results of PLS regression for three preprocessing methods

Preprocessing	LV number	R^2 Cal	R^2 Val	RMSEP
Mean Center	5	0.929	0.777	1.020
	6	0.959	0.803	0.882
Auto Scale	5	0.957	0.769	1.240
	6	0.979	0.788	1.040
Normalize + Mean Center	5	0.933	0.758	2.070
	6	0.983	0.859	0.831

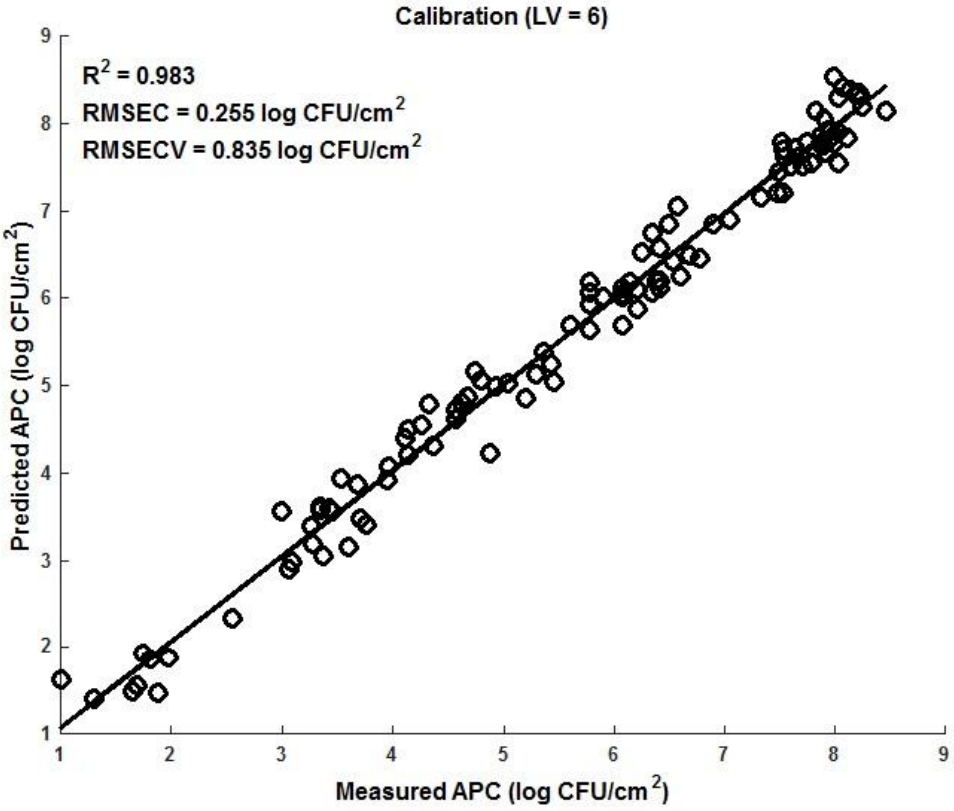

Figure 4-5. Predicted vs measured log (APC) obtained by PLSR calibration

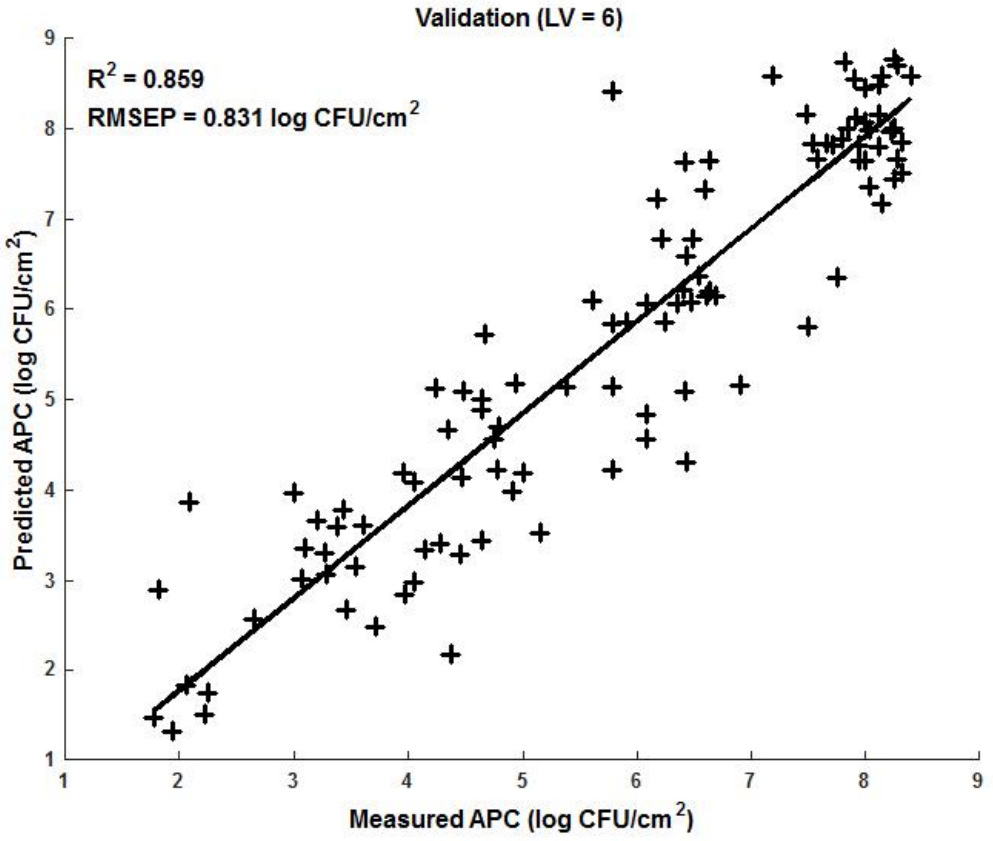

Figure 4-6. Predicted vs measured log(APC) obtained by PLSR validation

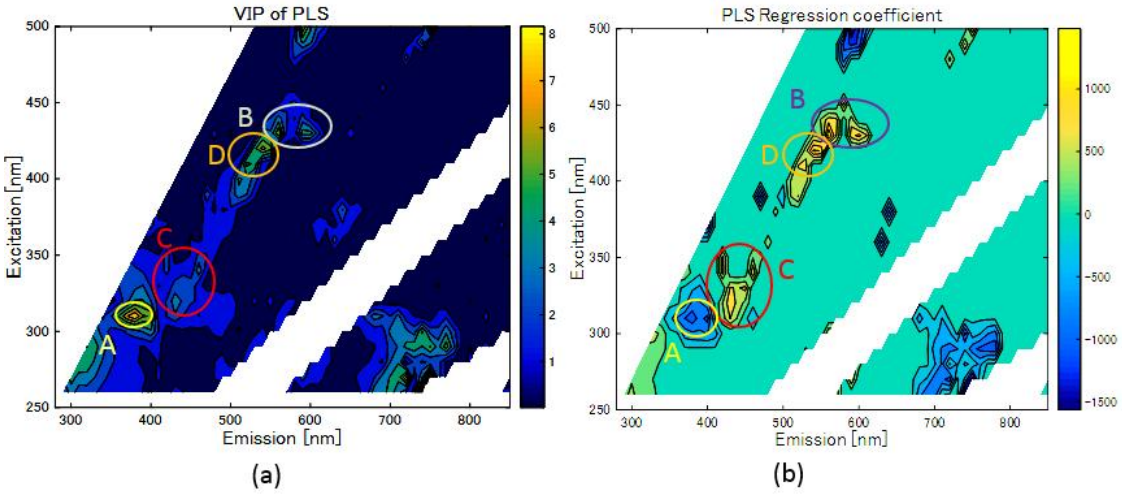

Figure 4-7. VIP and regression coefficient

(a) VIP score of PLS model; (b) Regression coefficient of PLS model
A:Tryptophan; B: Porphyrins; C: NAD(P)H; D: Phospholipid

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Chapter 5. Conclusion and Future Perspectives

5.1 Conclusions

The main achievements of this study are summarized as follow.

1. The FF spectroscopy system could predict *E. coli* cells in solutions with error of prediction were 0.79 CFU/ml.

2. The FF spectroscopy was applied to predict bacterial load on beef and pork surface. FF data were obtained from the metabolites trace.

3. A novel fluorescence spectroscopy measurement method using fiber optics which is rapid and contactless was developed. The FF with fiber optics system consist of the following points:

    a. FF with fiber optics consists of fluorescence spectrophotometer, fiber optics, and XY stage controller.

    b. Two set of program. Program based on Matlab for extracting and analysis FF data and program based on LabView for controlling XY stage controller.

    c. PLSR model for prediction of APC on the surface of beef stored at 15 ^{o}C.

4. Three different approaches (Bacterial cells, SDF, or Fiber-optics) for the prediction of

microbial load acquired by FF was compared to that of conventional APC method. The FF method was proved comparable to conventional method with the low error prediction.

5. This study found the important fluorophores for prediction of microbial load i.e., Tryptophan, Porphyrins, Flavins, and NAD(P)H.

6. The prediction of microbial load acquired by FF was rapid; it takes only about 2 minutes for FF measurement. Much faster than conventional method which takes several days for APC measurement.

5.2 Future Perspectives

There is scope for many studies regarding the development and application of the FF method for monitoring muscle food quality. Below are some future perspectives:

1. Portable FF measurement system could be developed. With the focus on monitoring or inspection. Portable equipment that is mobile, light, and economically affordable is necessary.

2. Aside from microbial content monitoring, adulteration is another problem in meat industries. Beef contaminated with horse meat, beef contaminated with pork, were not the only problem for the religious view but also for health perspectives. FF could be a novel method for monitoring these cases with superiority in easy, rapid, non-destructive.

3. The method could be applied to other samples. In my home country, Indonesia, a lot of food-borne illness cases is not only related to microbial but also food additives. Non-food grade dye, non-food preservative, and non-food thickener are just a few examples to be mentioned as additives that may be monitored by FF system.

As mentioned above, the study of the FF spectroscopy method has many possibilities. Fortunately, we have the chance of challenging these possibilities and hope to make the most of it.

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Appendix 1.

Matlab Scripts.

Scripts. 1 EEM overview

```
%% Extract EEM data from XLS folder

%% Delete Scattering Light

Beef_48H_delscat = FCN_EEM_DelScat (WL_Ex, WL_Em, Beef_48H_new);



% convert to log scale

Beef_48H_delscat_log = Beef_48H_log = log10(Beef_48H_new);



%% Making figure

figure;

contourf(WL_Em, WL_Ex, Beef_72H_log', 300);

shading flat; grid on;

caxis([0 4]); colorbar;

xlabel('Em WL [nm]'); ylabel('Ex WL [nm]');

title('Beef_72H', 'interpreter', 'none');
```

```
figure;

contourf(WL_Em, WL_Ex, Beef_48H_delscat_log', 300);

shading flat; grid on;

caxis([0 4]); colorbar;

title('Pork_48H_Log', 'interpreter', 'none');


%% Get Emission Spectra and put in array data

Beef_00H_log = log10(Beef_00H);

Beef_12H_log = log10(Beef_12H);

Beef_24H_log = log10(Beef_24H);

Beef_36H_log = log10(Beef_36H);

Beef_48H_log = log10(Beef_48H);

Beef_60H_log = log10(Beef_60H);

Beef_72H_log = log10(Beef_72H);


% tryptophan

Beef_00H_Ex290 = Beef_00H(:, 19);

Beef_12H_Ex290 = Beef_12H(:, 19);

Beef_24H_Ex290 = Beef_24H(:, 19);
```

```
Beef_36H_Ex290 = Beef_36H(:, 19);

Beef_48H_Ex290 = Beef_48H(:, 19);

Beef_60H_Ex290 = Beef_60H(:, 19);

Beef_72H_Ex290 = Beef_72H(:, 19);



Beef_00H_Ex290_log = Beef_00H_log(:, 19);

Beef_12H_Ex290_log = Beef_12H_log(:, 19);

Beef_24H_Ex290_log = Beef_24H_log(:, 19);

Beef_36H_Ex290_log = Beef_36H_log(:, 19);

Beef_48H_Ex290_log = Beef_48H_log(:, 19);

Beef_60H_Ex290_log = Beef_60H_log(:, 19);

Beef_72H_Ex290_log = Beef_72H_log(:, 19);



% making figure

figure;

plot (WL_Em, Beef_00H_Ex290_log, WL_Em, Beef_12H_Ex290_log, WL_Em,

Beef_60H_Ex290_log, WL_Em, Beef_72H_Ex290_log);

title('Beef_Ex290', 'interpreter', 'none');

xlabel('Em Wavelength [nm]'); ylabel('Intensity [a.u.]');
```

```
legend('Beef¥_00H¥_Ex290','Beef¥_12H¥_Ex290','Beef¥_60H¥_Ex290',
'Beef¥_72H¥_Ex290');

% NADH ex 320 em 440
Beef_00H_Ex320 = Beef_00H_log(:, 25);
Beef_12H_Ex320 = Beef_12H_log(:, 25);
Beef_24H_Ex320 = Beef_24H_log(:, 25);
Beef_36H_Ex320 = Beef_36H_log(:, 25);
Beef_48H_Ex320 = Beef_48H_log(:, 25);
Beef_60H_Ex320 = Beef_60H_log(:, 25);
Beef_72H_Ex320 = Beef_72H_log(:, 25);

figure;
plot (WL_Em, Beef_00H_Ex320, WL_Em, Beef_12H_Ex320, WL_Em, Beef_48H_Ex320,
WL_Em, Beef_72H_Ex320);
title('Beef_Ex320', 'interpreter', 'none');
xlabel('Em Wavelength [nm]'); ylabel('Intensity [a.u.]');
legend('Beef¥_00H¥_Ex320', 'Beef¥_12H¥_Ex320', 'Beef¥_48H¥_Ex320',
'Beef¥_72H¥_Ex320');
```

```
% Porphyrin ex 430 em 590 dan 660

Beef_00H_Ex430 = Beef_00H_log(:, 47);

Beef_12H_Ex430 = Beef_12H_log(:, 47);

Beef_24H_Ex430 = Beef_24H_log(:, 47);

Beef_36H_Ex430 = Beef_36H_log(:, 47);

Beef_48H_Ex430 = Beef_48H_log(:, 47);

Beef_60H_Ex430 = Beef_60H_log(:, 47);

Beef_72H_Ex430 = Beef_72H_log(:, 47);


figure;

plot (WL_Em, Beef_00H_Ex430, WL_Em, Beef_24H_Ex430, WL_Em, Beef_48H_Ex430,

WL_Em, Beef_72H_Ex430);

title('Beef_Ex430', 'interpreter', 'none');

xlabel('Em Wavelength [nm]'); ylabel('Intensity [a.u.]');

legend('Beef¥_00H¥_Ex430', 'Beef¥_24H¥_Ex430', 'Beef¥_48H¥_Ex430',

'Beef¥_72H¥_Ex430');


% Flavin ex 460 em 520
```

```
Beef_00H_Ex460 = Beef_00H_log(:, 53);

Beef_12H_Ex460 = Beef_12H_log(:, 53);

Beef_24H_Ex460 = Beef_24H_log(:, 53);

Beef_36H_Ex460 = Beef_36H_log(:, 53);

Beef_48H_Ex460 = Beef_48H_log(:, 53);

Beef_60H_Ex460 = Beef_60H_log(:, 53);

Beef_72H_Ex460 = Beef_72H_log(:, 53);



figure;

plot (WL_Em, Beef_00H_Ex460, WL_Em, Beef_48H_Ex460, WL_Em, Beef_60H_Ex460);

title('Beef_Ex460', 'interpreter', 'none');

xlabel('Em Wavelength [nm]'); ylabel('Intensity [a.u.]');

legend('Beef¥_00H¥_Ex460', 'Beef¥_48H¥_Ex460', 'Beef¥_60H¥_Ex460');
```

Script 2: Extract EEM for PLSR from Excel file

```
function extractEEM5b_IIL

% Load output files from spectrometer (F7000) in Excel format

% Extract EEM data, delete scattering light, reshape matrices and save



EEM_Origin = [];

EEM_Header = [];

fileNum = 0;



%% Select directory of output files from spectrometer (F7000)

folder = uigetdir('Select directory for EEM data');



%% List of files

files = dir(fullfile(folder, '*.xlsx'));

%disp('---');

%disp('Use following texts as the sample names in "EEM data.xlsx"');

fileNum = size(files, 1);

fnames = cell(fileNum,1);

% Extract file name
```

```
for i = 1:fileNum
    fnames{i} = regexprep(files(i,1).name, '.xlsx', '');
end;

%% Load EEM from each Excel file selected

for i = 1:fileNum
    data = xlsread(fullfile(folder, files(i).name));
    index = find(data(:,1)>=200, 1);
    eem = data(index-1:end, :);
    % Read EEM data
    [Ex, Em, tempEEM] = readEEM(eem);
    if i==1
        % Size of Ex & Em wavelength condition
        ExNum = max(size(Ex));
        EmNum = max(size(Em));
        EEM_Origin = zeros(ExNum*EmNum, fileNum);
    end
    % Reshape
```

```
    tempEEM = reshape(tempEEM, ExNum*EmNum, 1);
    % Conbination of matrices
    EEM_Origin(:,i) = tempEEM;
end


%% Create wavelength index
Ex = Ex';
wavelength = zeros(ExNum*EmNum, 2);
for i = 1:ExNum
    for j = 1:EmNum
        wavelength((i-1)*EmNum+j,:) = [Ex(i,:) Em(j,:)];
    end
end


%% Create wavelength index with header
wavelength_Header = cell(ExNum*EmNum, 3);
for i = 1:ExNum
    for j = 1:EmNum
        ExEm = ['Ex', num2str(Ex(i)), 'Em', num2str(Em(j))];
```

```
            wavelength_Header{(i-1)*EmNum+j, 1} = ExEm;
            wavelength_Header{(i-1)*EmNum+j, 2} = num2str(Ex(i));
            wavelength_Header{(i-1)*EmNum+j, 3} = num2str(Em(j));
        end
    end
    wavelength_Header = cat(1, {'ExEm' 'Ex' 'Em'}, wavelength_Header);

    %% Concatenate wavelength index with EEM data
    EEM_Header = num2cell(EEM_Origin);
    EEM_Header = cat(1, fnames', EEM_Header);
    EEM_Header = [wavelength_Header EEM_Header];

    EEM_Origin = [wavelength EEM_Origin];

    %% Save EEM data
    xlswrite(fullfile(folder, 'EEM_data.xlsx'), EEM_Header);

    %% Delete scattering light
    EEM_DelScat = delScattering3b_IIL(EEM_Origin, fnames);
```

```
%% Save processed EEM data

xlswrite(fullfile(folder, 'EEM_data_DelScat.xlsx'), EEM_DelScat);
```

Script 3: Extract EEM for PLSR from TXT file

```
%% Opening folder

folder = uigetdir(pwd,'Pilih EEM data folderB');


%File List

files=dir(fullfile(folder,'*.txt'));


fileNum=size(files,1);

fnames = cell(fileNum,1);

%File List

files=dir(fullfile(folder,'*.txt'));

fileNum=size(files,1);

sampleNumber=nan(fileNum,1);

fnames = cell(fileNum,1);


for i=1:fileNum
```

```
    tmpStr=files(i).name;
    pos=strfind(tmpStr,'_');
    sampleNumber(i)=str2num(tmpStr(pos(1)+1:pos(2)-2));
end
[tmp,sortIdx]=sort(sampleNumber);
files_sorted=files(sortIdx);
files=files_sorted;

for i = 1:fileNum
   pos=strfind(files(i,1).name,'FD3');
   fnames{i} = files(i,1).name([1:pos-2 pos+8:end]);

    %Read data as string
    fid=fopen(fullfile(folder,files(i).name));
    textData=textscan(fid,'%s','delimiter', '¥n');
    fclose(fid);

    %Fine Last line part
    textSize=size(textData{1});
```

```
for j=1:textSize(1)
    tmpText=textData{1}{j};
    if strcmp(tmpText,'ÃÞ°ÀØ½Ä')||strcmp(tmpText,'Data Points')
        dataStart=j;
        break
    end
end

%EEMCreate and save
data=dlmread(fullfile(folder,files(i).name),'¥t',dataStart,0);
fname=regexprep(files(i).name,'.txt','');
disp(fname);

 %EEM data read
tempEEM=data(2:end,2:end);

if i==1
    %waveleng information
    Ex=data(1,2:end);
```

```
    Em=data(2:end,1);

    ExNum=max(size(Ex));

    EmNum=max(size(Em));


    %variable initiation

    outputEEM=NaN(EmNum,ExNum);

    EEM_Origin = zeros(ExNum*EmNum, fileNum);

    % sName=cell(fileNum,1);

end


    %Reshape

    tempEEM = reshape(tempEEM, ExNum*EmNum, 1);

     % Conbination of matrices

    EEM_Origin(:,i) = tempEEM;



    % outputEEM(:,:)=tempEEM;

    % destFile = strcat(fnames{i}, '.xlsx') ;

    % xlswrite(fullfile(folder, destFile), outputEEM);
```

```
end;
%% Create wavelength index
Ex = Ex';
wavelength = zeros(ExNum*EmNum, 2);
for i = 1:ExNum
    for j = 1:EmNum
        wavelength((i-1)*EmNum+j,:) = [Ex(i,:) Em(j,:)];
    end
end
%% Create wavelength index with header
wavelength_Header = cell(ExNum*EmNum, 3);
for i = 1:ExNum
    for j = 1:EmNum
        ExEm = ['Ex', num2str(Ex(i)), 'Em', num2str(Em(j))];
        wavelength_Header{(i-1)*EmNum+j, 1} = ExEm;
        wavelength_Header{(i-1)*EmNum+j, 2} = num2str(Ex(i));
        wavelength_Header{(i-1)*EmNum+j, 3} = num2str(Em(j));
    end
```

```
end

wavelength_Header = cat(1, {'ExEm' 'Ex' 'Em'}, wavelength_Header);


%% Concatenate wavelength index with EEM data

EEM_Header = num2cell(EEM_Origin);

EEM_Header = cat(1, fnames', EEM_Header);

EEM_Header = [wavelength_Header EEM_Header];

EEM_Origin = [wavelength EEM_Origin];


%% Save EEM data

xlswrite(fullfile(folder, 'EEM_data.xlsx'), EEM_Header);


%% Delete scattering light

EEM_DelScat = delScattering3b_IIL(EEM_Origin, fnames);


%% Save processed EEM data

xlswrite(fullfile(folder, 'EEM_data_DelScat.xlsx'), EEM_DelScat);
```

Script 4: making dataset

```
% Dataset load script


%% Dataset of Aerobic Plate Count

Calib_APC = dataset;

Calib_APC.name = 'APC';

Calib_APC.data = APC_mix;

Calib_APC.label{1} = Calib_SampleName;

Calib_APC.labelname{1} = 'Sample';

Calib_APC.label{2} = 'APC[CFU/cm2]';

Calib_APC.labelname{2} = 'APC[CFU/cm2]';


Calib_APC_Log = Calib_APC;

Calib_APC_Log.name = 'log(APC)';

Calib_APC_Log.data = log10(Calib_APC.data);

Calib_APC_Log.label{2} = 'log(APC[CFU/cm2])';

Calib_APC_Log.labelname{2} = 'log(APC[CFU/cm2])';


%% Dataset of EEM for Calibration
```

```
%buat EEMVarName = {}, lalu paste excel data

Calib_EEM = dataset;

Calib_EEM.name = 'EEM data';

Calib_EEM.data = EEM_Calib;

Calib_EEM.label{1} = Calib_SampleName;

Calib_EEM.labelname{1} = 'Sample';

Calib_EEM.label{2} = EEMVarName(:,1);

Calib_EEM.labelname{2} = 'Ex/Em Wavelength [nm]';



Calib_EEM_Log = Calib_EEM;

Calib_EEM_Log.name = 'log(EEM) data for Calibration';

Calib_EEM_Log.data = log10(Calib_EEM_Log.data);



% Dataset load script

%% Storage & class_id

tmpNum=size(Calib_SampleName,1);

Storage=cell(tmpNum,1);



for i=1:tmpNum
```

```
    tmpStr=Calib_SampleName{i};
    pos=strfind(tmpStr,'_');
    tmpSto=tmpStr(pos(1)+1:pos(2)-2);
    Storage{i}= tmpSto;
end

clear i;
clear pos;
clear tmpNum;
clear tmpSto;
clear tmpStr;

%% making class on dataset

Calib_EEM.classid{1,1}=Storage;
```

Script 4: making PLSR and coefficients regression figure

```
Function Figure_PLSModel_CaliVali_LinearReg_RegCon(PLSModel_Cali, PLSModel_Vali,
EEM_VarName)
```

```
%load PLS Model

LV = size(PLSModel_Cali.wts, 2);

CaliMeasY = double(PLSModel_Cali.detail.data{2});

CaliPredY = cell2mat(PLSModel_Cali.pred(2));

ValiMeasY = double(PLSModel_Vali.detail.data{2});

ValiPredY = cell2mat(PLSModel_Vali.pred(2));

[P1, CaliFitY, CaliR2] = LinearRegression_Func(CaliMeasY, CaliPredY);

[P2, ValiFitY, ValiR2] = LinearRegression_Func(ValiMeasY, ValiPredY);



% Figure for PLS

figure; hold on;

plot(CaliMeasY, CaliPredY, 'LineStyle', 'none', 'Marker', 'o', 'Color', 'b', 'MarkerSize', 8);

plot(CaliMeasY, CaliFitY, 'Color', 'r', 'LineWidth', 1);

title(['Calibration (LV = ' num2str(LV) ')'], 'FontSize', 12);

set(gca, 'FontSize', 10);

% axis([4 8 4 8]);

% text(4.5, 7.5,'text');

Axis1 = axis;
```

```
Str_RegFunc1 = [num2str(P1(1)) ' * X + ' num2str(P1(2))];

Str_CaliR2 = ['R^2 = ' num2str(CaliR2)];

Str_RMSEC = ['RMSEC = ' num2str(PLSModel_Cali.detail.rmsec(LV))];

Str_RMSECV = ['RMSECV = ' num2str(PLSModel_Cali.detail.rmsecv(LV))];

Str_CaliBias = ['Cali Bias = ' num2str(PLSModel_Cali.detail.bias(LV))];

text(Axis1(1)+0.2, Axis1(4)-0.2,...

   {Str_RegFunc1, Str_CaliR2, Str_RMSEC, Str_RMSECV, Str_CaliBias},...

   'VerticalAlignment', 'Top', 'HorizontalAlignment', 'Left',...

   'FontSize', 12);

xlabel('Measured Y', 'FontSize', 12);

ylabel('Predicted Y', 'FontSize', 12);

hold off;



% Validation figure

figure; hold on;

plot(ValiMeasY, ValiPredY, 'LineStyle', 'none', 'Marker', '+', 'Color', 'b', 'MarkerSize', 8);

plot(ValiMeasY, ValiFitY, 'Color', 'r', 'LineWidth', 1);

title(['Validation (LV = ' num2str(LV) ')'], 'FontSize', 12);

set(gca, 'FontSize', 10);
```

```
% axis([4 8 4 8]);

% text(4.5, 7.5,'text');

Axis2 = axis;

Str_RegFunc2 = [num2str(P2(1)) ' * X + ' num2str(P2(2))];

Str_ValiR2 = ['R^2 = ' num2str(ValiR2)];

Str_RMSEP = ['RMSEP = ' num2str(PLSModel_Vali.detail.rmsep(LV))];

Str_ValiBias = ['Vali Bias = ' num2str(PLSModel_Vali.detail.bias(LV))];

text(Axis2(1)+0.2, Axis2(4)-0.2,...

   {Str_RegFunc2, Str_ValiR2, Str_RMSEP, Str_ValiBias},...

   'VerticalAlignment', 'Top', 'HorizontalAlignment', 'Left',...

   'FontSize', 12);

xlabel('Measured Y', 'FontSize', 12);

ylabel('Predicted Y', 'FontSize', 12);

hold off;



% PLS coefficient

Ex = [200:10:900]';

Em = [200:10:900];

RegConData = [cell2mat(EEM_VarName(:,2)) cell2mat(EEM_VarName(:,3))
```

```
PLSModel_Cali.reg];

PLS_Coeff = NaN(71, 71);

DataNum = size(RegConData,1);

for i = 1:DataNum

   IndexEx = find(Ex == RegConData(i,1));

   IndexEm = find(Em == RegConData(i,2));

   PLS_Coeff(IndexEx, IndexEm) = RegConData(i,3);

end;

figure;

set(gca, 'FontSize', 10);

contourf(Ex, Em, PLS_Coeff, 30);

shading flat;

title('PLS Regression Coefficient', 'FontSize', 12);

xlabel('Em [nm]', 'FontSize', 12);

ylabel('Ex [nm]', 'FontSize', 12);

axis([200 900 200 900]);

set(gca, 'FontSize', 10);

colorbar;

grid on; end
```